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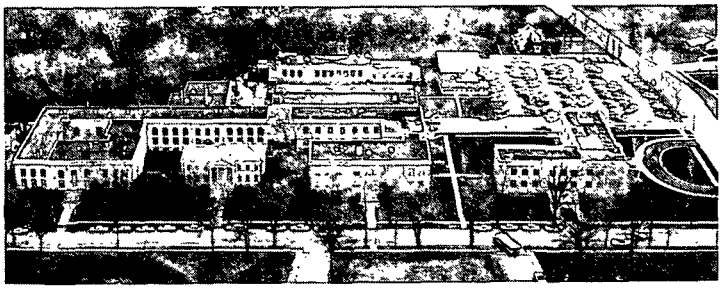

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CHLOROPLAST ULTRASTRUCTURE AND GENE EXPRESSION IN EMBRYOGENIC CONIFER CALLUS

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Chloroplast ultrastructure and gene expression in embryogenic conifer callus

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Abstract

Embryogenic conifer calli are characteristically white or translucent in appearance even when grown in the light. Ultrastructural examination of embryogenic Norway spruce callus has revealed that somatic embryos contain plastids resembling the proplastids found in zygotic embryos. Mature chloroplasts, containing grana, are found in nonembryogenic callus, which is green when grown under identical conditions. Proplastids are also found in embryogenic calli of other conifer species. The morphology of the plastids change as the somatic embryos develop. Soluble proteins extracted from both callus types have been compared by SDS-PAGE. Differences in proteins extracted from the two tissue types have been observed, some of which may relate to chloroplast development/ultrastructure.

Introduction

Perhaps the most distinguishing feature of embryogenic Norway spruce callus is its translucent appearance, even when grown in the light (5). Non-embryogenic Norway spruce callus is characteristically green when grown under these conditions. Embryogenic calli of larch, Douglas-fir, white pine, loblolly pine and pond pine also exhibit this white or translucent phenotype (3,4,15). In addition to biochemical studies of the two callus types (14), ultrastructural and molecular examinations of the cells are being carried out to better characterize embryogenic conifer callus. We wished to determine if the lack of green coloration of the embryogenic calli was due to a deficiency of chlorophyll within chloroplasts, the absence of chloroplasts, or the presence of an alternate form of plastid such as a proplastid which is typically found in early zygotic embryos or meristems.

Mature chloroplasts arise from progenitor plastids in meristems and embryos via a defined developmental pathway progressing from eoplasts, amyloplasts, amoeboid plastids (termed proplastids) to mature chloroplasts. Although the terminology and order of the progression through the proplastid forms is subject to some variation in the literature, the general scheme outlined above appears to be well accepted (13). Our ultrastructural studies of cultured and intact tissues have concentrated on the morphology of plastids. To date, chloroplasts (plastids) in all of the above mentioned conifer calli (green and white calli) have been examined. Zygotic and somatic embryos of several species have also been examined. Soluble proteins were separated by SDS-PAGE and visualized by silver staining, and relative levels of the mRNA encoding the small subunit of ribulose 1,5-bisphosphate carboxylase (rubisco) were compared to determine differences in the expression of a protein found in the chloroplasts of embryogenic

and nonembryogenic calli. Embryogenic conifer calli contain chloroplasts (plastids) that differ from mature chloroplasts not only morphologically, as illustrated by microscopic examinations, but also biochemically as suggested by SDS-PAGE and other gene expression studies.

Materials and Methods

Cultures of embryogenic and nonembryogenic conifer callus are available as part of the ongoing work of the tissue culture project at The Institute of Paper Chemistry. Cultured tissues included Picea abies (Norway spruce), Picea glauca (white spruce), Larix decidua (European larch), Pinus taeda (loblolly pine), Pinus strobus (white pine), Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) and Pinus serotina Michx. (pond pine) that were generally initiated from embryos as previously described (4,5,15).

Samples were prepared for transmission electron microscopy (TEM) as follows: Tissue was fixed in 3% glutaraldehyde in 0.05M sodium cacodylate buffer, pH 7.0 (also containing 0.05% calcium chloride and 0.1M glucose) for 2-3 h, washed briefly with buffer, and postfixed in 1% OsO_4 in the same buffer for 2-3 h at room temperature. After a brief wash, samples were slowly dehydrated in ethanol at 4°C, followed by slow infiltration with SPURR at room temperature, and embedded by polymerization at 70°C for 6 h. The tissues were sectioned at 90 nm and stained with uranyl acetate [5% in 50% (v/v) ethanol] followed by lead citrate (0.5%, pH 12). To ensure penetration/infiltration of fixative into seedling cotyledon sections and zygotic embryos, the tissues were subjected to several cycles of vacuum/atmospheric pressure while in fixative.

Cotyledons were collected from seedlings germinated under normal greenhouse conditions (light grown) or in complete darkness (dark grown; all handling and

sample collection was performed under a dim green safelight) 2-3 weeks after the seeds were sown.

Total chlorophyll was estimated by determining the A652 of an 80% acetone extract of the tissues. The O.D. was then used to calculate µg chlorophyll (1).

Soluble proteins were extracted from embryogenic or nonembryogenic calli by homogenizing the tissue in cold 50 mM HEPES (pH = 7.5), 1 mM PMSF. After centrifuging for 5 min in an Eppendorf microfuge, protein in the supernatant was quantified by the Bradford dye binding assay and loaded onto standard 12% or 7.5-15% gradient SDS-polyacrylamide gels. Equal amounts of protein (2-3 µg) were loaded onto each lane and visualized in the gel using a modified silver stain technique.

Results and Discussion

When total chlorophyll levels were estimated in acetone extracts of the callus tissues, the results obtained were not surprising and simply confirmed, on a quantitative basis, visual observations that had been described earlier (Fig. 1).

The chloroplasts of light grown conifers are typical of those found in most higher plants. The organelles, usually 2 to 10 microns in length, contain internal thylakoid membranes organized into grana, occasional starch grains and protein bodies (Fig. 2a). The organization of the thylakoid membranes is dependent, however, upon the age and physiological state of the tissue (Fig. 2b).

The plastids in the embryogenic spruce callus exhibit a different morphology at the ultrastructural level. The plastids, which appear more darkly stained

(more electron dense) than mitochondria in the photographs, lack the internal organization of a fully developed chloroplast. At higher magnification some mottling is evident, which may reflect the presence of thylakoid membranes which are in a disorganized state (Fig. 3a,b). Some of these plastids contain small starch grains, although they are not nearly as large as the starch grains present in mature chloroplasts in leaf or cotyledon tissue. The plastids present in the green, nonembryogenic spruce tissues appear to be very similar to a typical chloroplast (Fig. 3c,d). Thylakoid membranes, some organized into grana, are found in the chloroplasts. Large starch grains are also prominent in some of the chloroplasts.

Zygotic embryos excised from immature loblolly pine seeds were also examined by TEM. As shown in Fig. 4, plastids lacking organized thylakoid membranes are abundant. Although a significant number contain starch grains, they resemble the plastids observed in embryogenic spruce calli.

In order to determine if the plastid morphology observed in somatic embryos was unique to Norway spruce, embryogenic callus of larch, loblolly pine, pond pine, white pine and Douglas-fir were also examined. Embryogenic callus of these conifer species contain plastids that closely resemble those in the embryogenic Norway spruce calli (Fig. 5,6). Irregularly shaped plastids lacking a significant degree of thylakoid membrane organization or grana formation were noted. Nonembryogenic calli of these species generally contained well developed chloroplasts, very similar to those seen in nonembryogenic Norway spruce calli. The plastids observed in somatic embryos of carrot, a model system in which development is well characterized, exhibit the same morphology as both the embryogenic conifer calli (data not shown). In most higher plants, including conifers, several aspects of chloroplast development are regulated by light.

Progenitors of chloroplasts, proplastids, are stimulated by light during germination to form mature chloroplasts. If seeds are germinated in the dark or if other tissues are grown in the darkness, chloroplast development may be altered such that etioplasts are formed (Fig. 2b). Etioplasts are colorless, hence the tissues do not appear green, and contain thylakoid membranes that are not organized in grana. A prominent prolamellar body is normally present, which may represent a site of temporary storage of the thylakoid membrane components. Upon further development, such as that stimulated by light, thylakoid membranes are formed and appear to radiate from the prolamellar bodies to form grana. It is obvious that the chloroplasts in dark grown, pale colored Norway spruce and other conifer seedlings (data not shown) are notably different from those in the pale or translucent, embryogenic calli. Clearly the proplastids in the embryogenic calli are not due to a simple lesion in the light induced development of the mature chloroplast.

The plastids observed in our tissues closely resemble descriptions and photographs of proplastids found in developing zygotic embryos. In perhaps the most thorough ultrastructural examination of zygotic embryogenesis reported in the literature, the embryonic development of Capsella (shepherd's purse) was followed from the egg, 2-cell, 3-cell, heart and torpedo stages through the mature embryo (10,11). In these reports, proplastids in the one-cell zygote through the heart stage embryos were found to contain proplastids that appear very similar to those in our tissues. Zygotic embryos of black pine (P. nigra) and jack pine (P. banksiana) have been examined at the ultrastructural level (2,8), although the authors stated that proplastids were difficult to recognize before germination or simply identified the plastids as proplastids and did not elaborate on their presence or significance. Proplastids similar to those noted

in our embryogenic calli and zygotic embryos are evident in the micrographs in both of these reports, however.

Relatively few reports describing ultrastructural studies of cultured plant tissues exist in the literature, and only a handful of these include observations of plastid or chloroplast morphology. Chloroplast differentiation has been observed in cultured tobacco and carrot calli, in which the morphology of the plastids were noted to change from chromoplasts and leukoplasts to fully developed chloroplasts (6,12). However, tissues examined in these studies were not from somatic embryos, but instead included proliferating callus. Some of the plastids in these reports did appear to be similar to those found in our embryogenic callus. Recently, Profumo et al. (9) described ultrastructural differences between embryogenic and nonembryogenic callus of Aesculus. Undifferentiated plastids or plastids devoid of an inner membrane system were noted in their embryogenic callus.

The proteins separated and visualized by SDS-PAGE showed clear differences between the embryogenic and nonembryogenic calli, with the exception of white spruce (Fig. 7). White spruce also failed to exhibit other biochemical differences which are used to characterize the two callus types (unpublished data, The Institute of Paper Chemistry). The most obvious difference observed on the protein gels concerns the presence of a very prominent protein of approximately 18-20 kd. This protein is interesting not only because it appears to be abundant in the nonembryogenic tissues, but also because it appears to be the same size in all the species examined (excepting white spruce). The abundance and size of the protein suggests that the protein could be an unprocessed form of the small subunit of rubisco. The small subunit of rubisco, a highly conserved protein encoded in nuclear DNA, is typically processed upon import into the

chloroplast to remove a ~5 kd transit peptide to yield a mature protein of ~15 kd. The abundant protein in our preparations is about 5 kd larger than mature rubisco small subunit (as identified by co-electrophoresis with a major band present in extracts of light grown cotyledons of larch seedlings), suggesting that the abundant protein may be the small subunit precursor. It must be noted, however, that small subunit precursor rarely, if ever, accumulates in vivo. Preliminary RNA dot blot data (data not shown), generated using a ss-rubisco probe isolated from a larch cDNA library, suggests that rubisco small subunit transcript levels are higher in the nonembryogenic callus as compared to the embryogenic callus. This finding would support the hypothesis that the abundant band in the nonembryogenic protein preparations is rubisco small subunit, but the RNA dot blot and a Northern blot need to be repeated in order to confirm this hypothesis.

In conclusion, it appears that proplastids or undifferentiated plastids are present in the embryogenic conifer calli, while fully developed chloroplasts are found in the green, nonembryogenic calli. Further work planned in our lab involves an attempt to identify the abundant protein found in the nonembryogenic calli, along with other ongoing studies designed to look at differences in gene expression in embryogenic and nonembryogenic tissues. Aside from their use as markers of conifer somatic embryogenesis, both the chloroplast ultrastructure and soluble protein differences also offer a valuable opportunity to study the differentiation/development of both conifer embryos and chloroplasts.

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Acknowledgments

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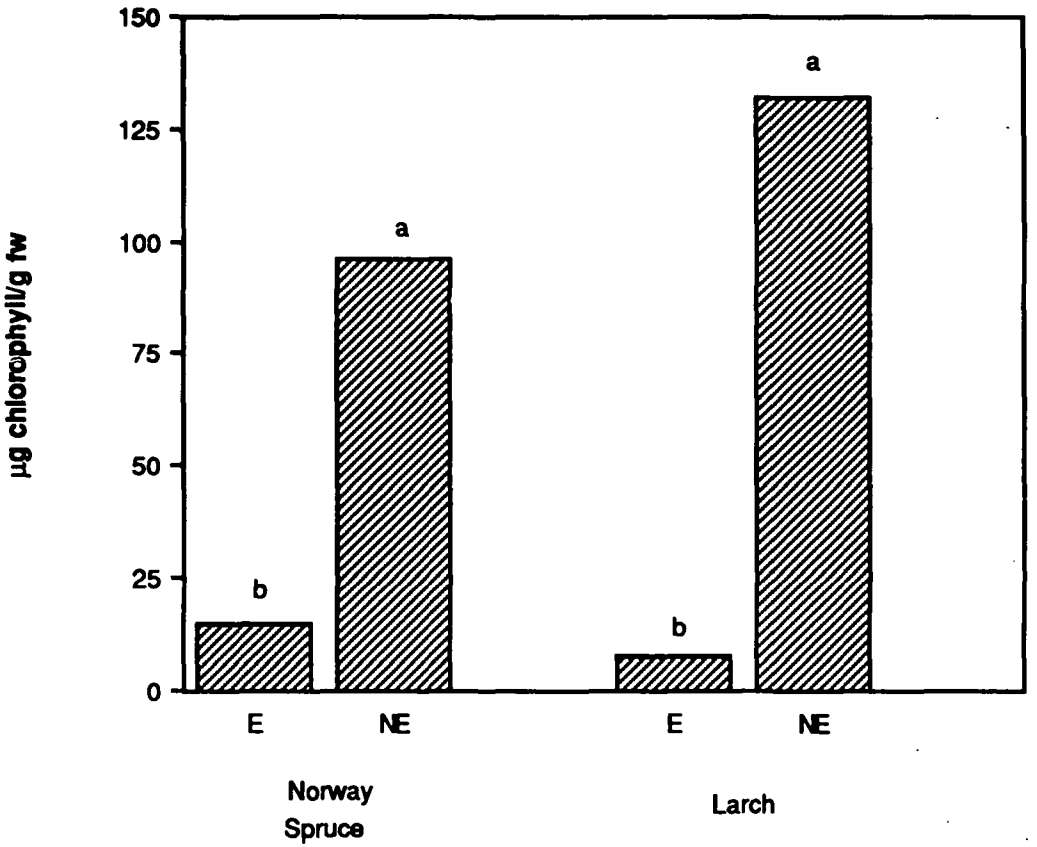

Figure 1. Chlorophyll levels in embryogenic (E) and nonembryogenic (NE) conifer calli.

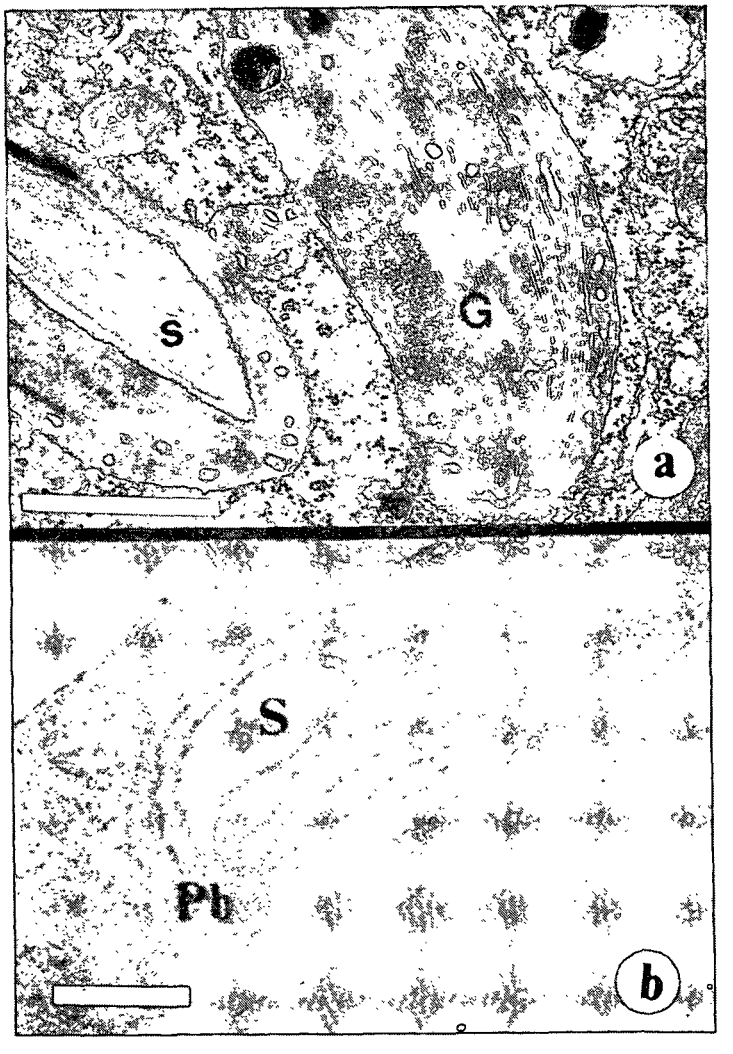

Figure 2. Typical chloroplasts found in green conifer tissue (taken from Douglas-fir nonembryogenic callus) (a) and a chloroplast present in dark grown Norway spruce seedling cotyledon (b).
Legend: bar = 1 micron, G = grana, M = mitochondrion, N = nucleus, P = proplastid, Pb = prolamellar body, S = starch grain.

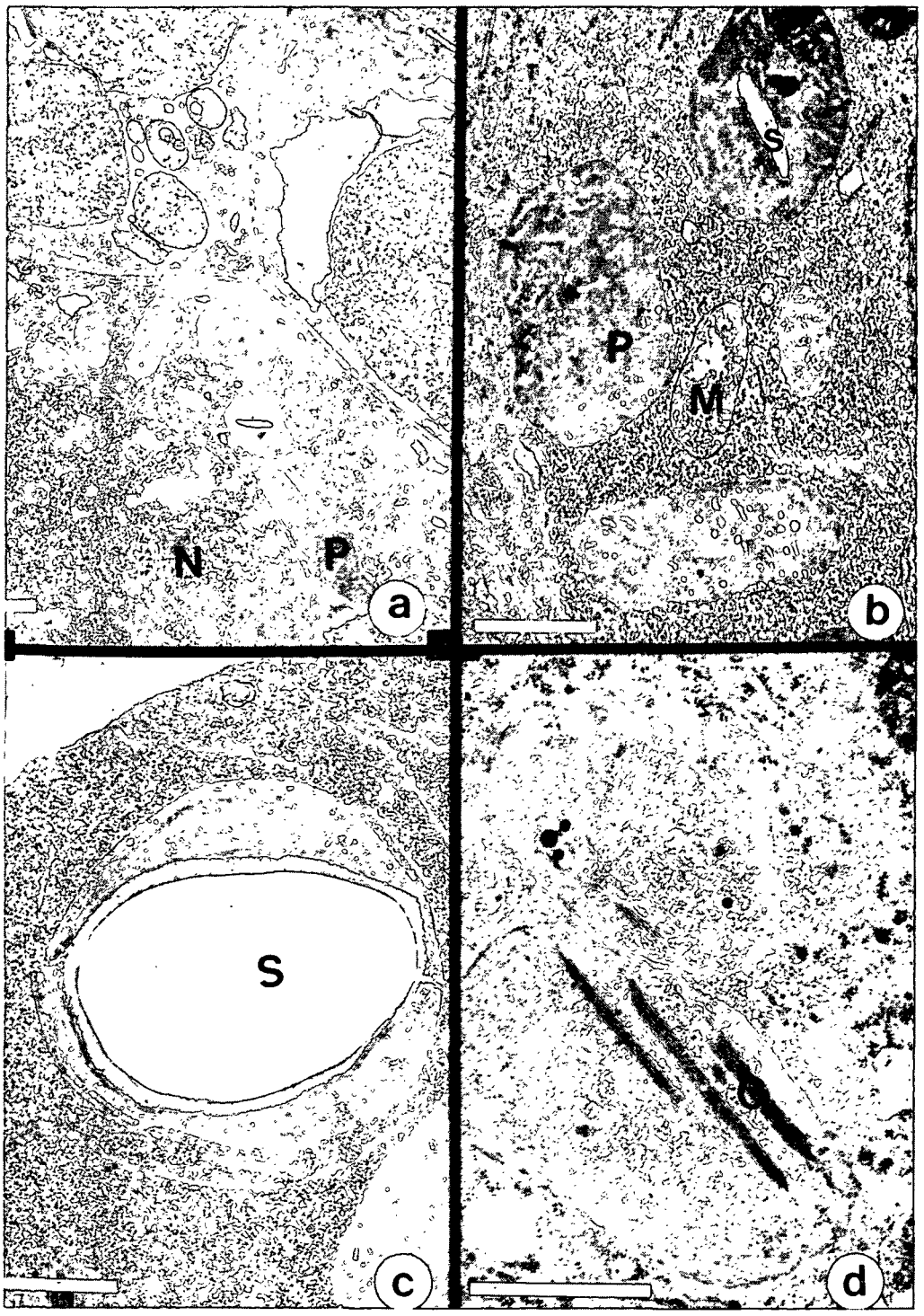

Figure 3. Plastids present in embryogenic (a,b) and nonembryogenic Norway spruce calli (c,d).

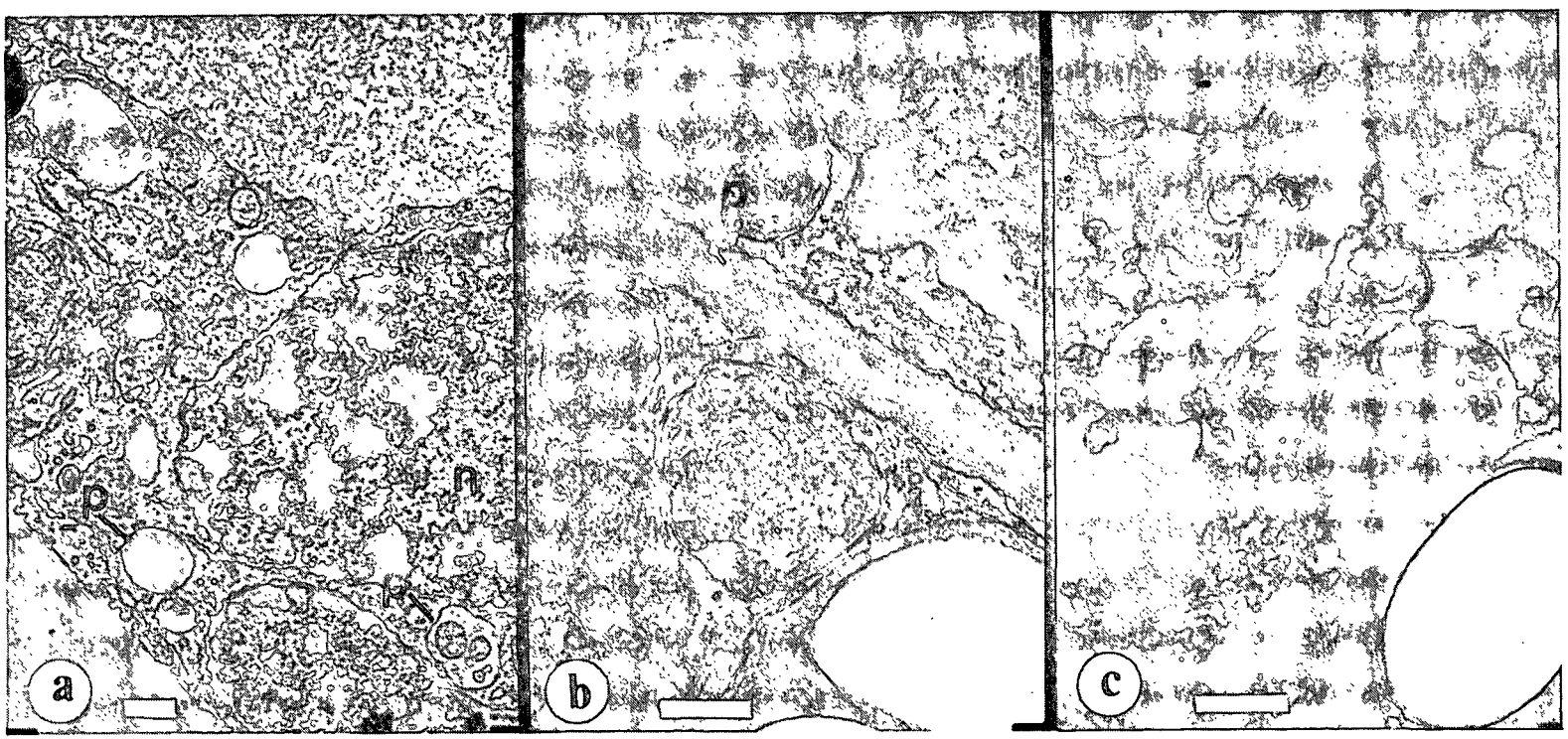

Figure 4. Plastids present in white pine (a), pond pine (b) and loblolly pine (c) embryogenic calli.

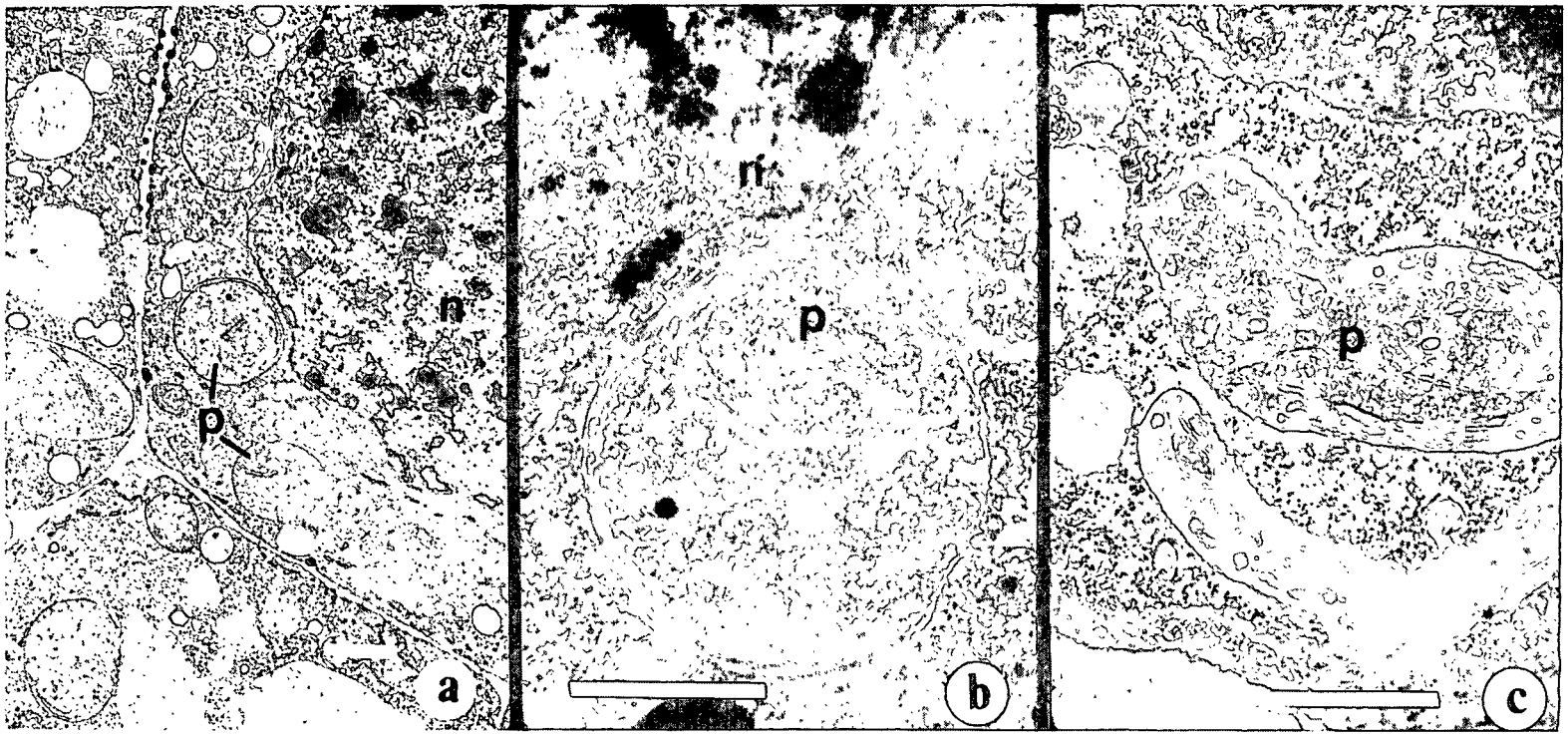

Figure 5. Plastids found in immature loblolly pine embryos.

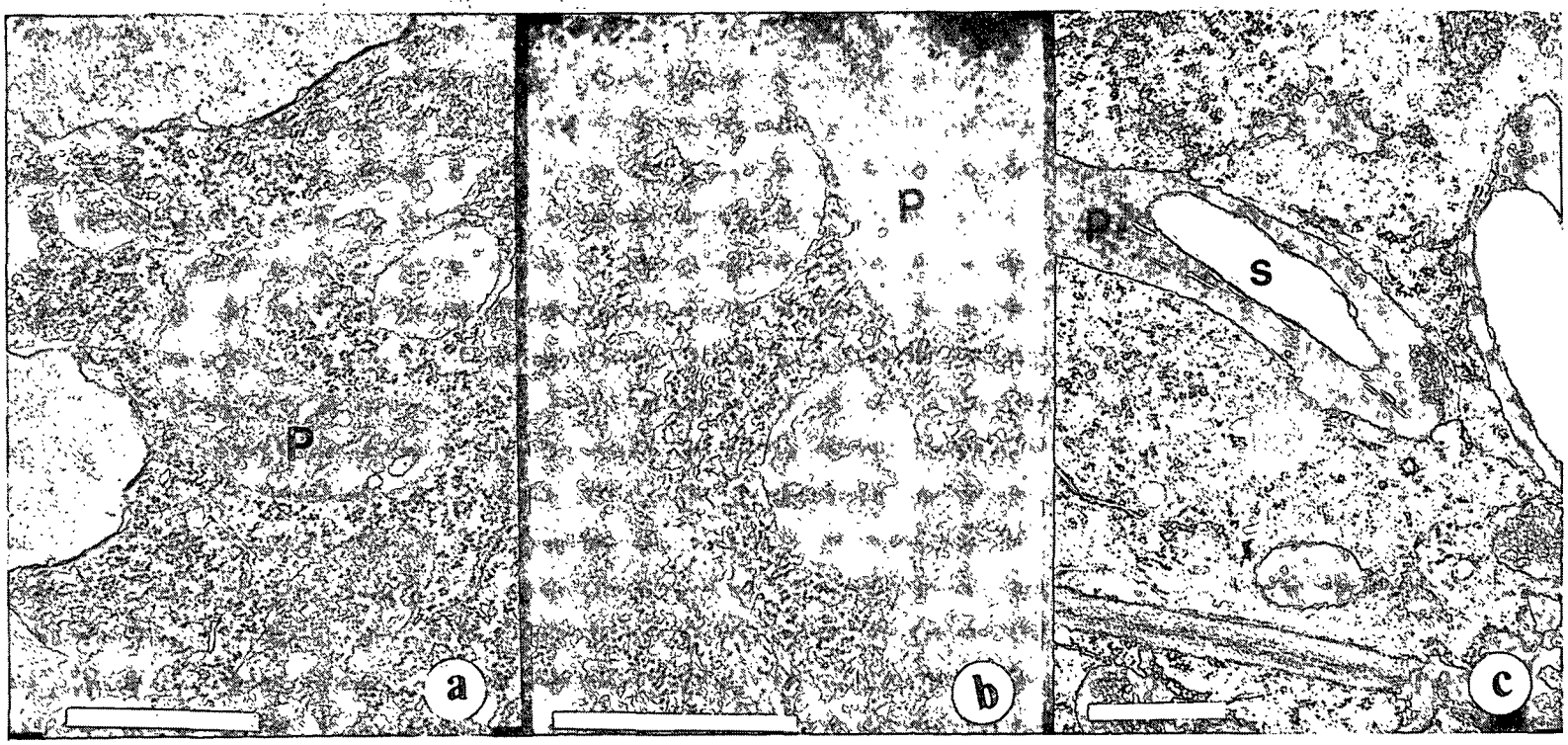

Figure 6. Plastids present in embryogenic (a,b) and nonembryogenic (c) larch calli.

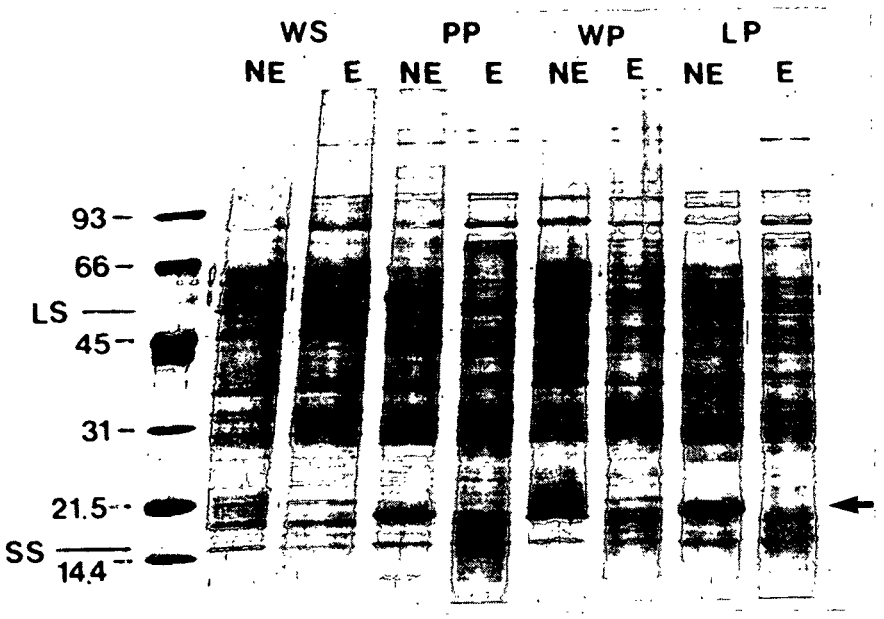

Figure 7. SDS-PAGE of soluble proteins extracted from white spruce (WS), pond pine (PP), white pine (WP) and loblolly pine (LP) embryogenic (E) and nonembryogenic (NE) calli.